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13. ABSTRACT *(Maximum 200 words)*

This research was intended to examine molecular aspects of sleep homeostasis in the brain. Our experiments were guided by the two-process model of sleep regulation which posits that an increased homeostatic "drive" to sleep occurs during prolonged wakefulness. We found that expression of brain-derived neurotrophic factor (BDNF) mRNA increases in the rat cortex during a 6 hr sleep deprivation period. Increased BDNF mRNA levels likely results in elevated expression of BDNF protein which we hypothesize may protect neurons from the potentially deleterious effects of prolonged sensory stimulation during wakefulness.

In the course of these studies, we cloned a novel gene, hypocretin, that encodes two biologically active neuropeptides expressed within a very restricted area of the posterior hypothalamus. When injected into the lateral venticles of the brain, the hypocretin peptides stimulate food intake, increase wakefulness and decrease deep slow wave sleep and REM sleep. In humans, the sleep disorder narcolepsy has recently been associated with degeneration of the hypocretin neurons. We have therefore proposed a model in which the hypocretins play a central role in arousal state (i.e., sleep/wake) regulation.

We also studied gene expression in the hibernating brain, as another model of arousal state.

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(4) STATEMENT OF THE PROBLEM STUDIED

The purpose of this proposal was to examine the biochemical and molecular aspects of sleep homeostasis in the brain. The experiments were guided by the two-process model of sleep regulation which posits that an increased homeostatic "drive" to sleep occurs during prolonged wakefulness. We hypothesize that (1) a molecular basis exists for the homeostatic regulation of sleep; (2) perturbations of the sleep homeostatic system result in compensatory changes in gene expression in brain that increase the likelihood of subsequent sleep; and (3) sleep after prolonged wakefulness involves a change in macromolecular synthesis that facilitates neuronal recovery or restoration.

In the course of pursuing this hypothesis, we identified a novel hypothalamic gene which encoded two previously undescribed neuropeptides. A large part of our effort during the funding period was devoted to studying the anatomy and physiology of this novel neurotransmitter system. The relevance of this work to sleep has been established by recent studies which indicate that ICV injections of the hypocretins promote wakefulness and decrease deep slow wave sleep and REM sleep and the discovery that, in humans, the sleep disorder narcolepsy is associated with degeneration of the hypocretin neurons.

(5) SUMMARY OF THE MOST IMPORTANT RESULTS

Sleep deprivation. During the course of this research, we found that expression of brain-derived neurotrophic factor (BDNF) mRNA increases in the cortex during a 6 hr sleep deprivation period (Peyron et al., 1998b). Increased BDNF mRNA levels in the cortex likely results in elevated expression of BDNF protein which we hypothesize may protect neurons from the potentially deleterious effects of sensory stimulation during wakefulness.

Isolation of the hypocretin gene. Using directional tag PCR subtractive hybridization (Kilduff et al., 1998), we isolated a novel neuropeptide gene, hypocretin, that encodes biologically active two peptides, hypocretin-1 and hypocretin-2 in a collaboration with Dr. Greg Sutcliffe at The Scripps Research Institute (de Lecea et al., 1998). *In situ* hybridization revealed that preprohypocretin was expressed exclusively by a bilaterally symmetric structure within the posterior hypothalamus. This hypothalamus–specific mRNA was the precursor of a pair of peptides which share substantial amino acid identities with the gut hormone secretin. Rat preprohypocretin encodes a 130 amino acid putative secretory protein with 4 sites for potential proteolytic maturation. Two of the putative products, which appear to be C–terminally amidated, share 14 identities across 20 amino acids. This region shares a 7/7 match with the gut hormone secretin. Mouse hypocretin (*Hcrt*) differs from rat at 7 amino acids. These differences obliterate 2 of the possible proteolytic products, but preserve the peptides that are related both to each other and to secretin, consistent with an evolutionarily conserved function for the 2 new peptides. The *Hcrt* gene, which in mouse is located on chromosome 11, is expressed most prominently after postnatal week 3.

In situ hybridization indicates that the neurons expressing preprohypocretin mRNA are located exclusively in the posterior hypothalamus. A polyclonal antiserum was raised in rabbits against the C-terminal 17-residues of preprohypocretin. We undertook an immunohistochemical study to determine the distribution of preprohypocretin-immunoreactive (preprohypocretin-IR neurons and fibers in the brain (Peyron et al., 1998a). In accord with the *in situ* hybridization data, preprohypocretin-IR cell bodies were observed exclusively in the perifornical nucleus and the dorsal and lateral hypothalamic areas. The fibers of these neurons are widespread throughout the posterior hypothalamus and project to multiple targets in other areas, including brainstem and thalamus. Preprohypocretin-IR fibers were located throughout the posterior hypothalamus, and in the preoptic area, the mediodorsal and reuniens nuclei of the thalamus, the dorsal raphe nucleus, the locus coeruleus, the laterodorsal tegmental nucleus, the central gray, the colliculi and the nucleus of the solitary tract. Few labeled fibers were located in cortical regions. These results indicate that preprohypocretin is translated as a peptide in the rat hypothalamus and transported to several brain areas.

At the EM level, preprohypocretin-IR is associated with large granular vesicles at synapses. One of the Hcrt peptides (hcrt-2) was excitatory when applied to cultured, synaptically coupled

hypothalamic neurons, but not hippocampal neurons (de Lecea et al., 1998). These observations suggest that the hypocretins function within the CNS as neurotransmitters. Indeed, another group subsequently cloned the same gene and the cognate receptors for the two peptides which they called the orexins because, when injected into the lateral venticles of the brain, these peptides stimulated food intake(Sakurai et al., 1998). We subsequently determined that one-third of all medial and lateral hypothalamic neurons tested, but not hippocampal neurons, showed a striking nanomolar sensitivity to hypocretin (van den Pol et al., 1998). As studied with calcium digital imaging with fura-2, hypocretin raised cytoplasmic calcium via a mechanism based on G-protein enhancement of calcium influx through plasma membrane channels. The peptide has a potent effect at both presynaptic and postsynaptic receptors. With whole-cell patch-clamp recording, we showed that hypocretin, acting directly at axon terminals, can increase the release of the amino acid transmitters GABA and glutamate. We also showed that Hcrt-2 regulates the synaptic activity of physiologically identified neuroendocrine neurons studied in hypothalamic slices containing the arcuate nucleus, suggesting a function of hypocretin in hormone regulation.

The most dense arborization of hypocretin axons in the brainstem was detected in the locus coeruleus (LC). In electrophysiological studies with slices of rat brain, we found that all LC cells showed excitatory responses to the hypocretin-2 peptide (Horvath et al., 1999). Hypocretin-2 uniformly increased the frequency of action potentials in these cells, even in the presence of tetrodotoxin, indicating that receptors responding to hypocretin were expressed in LC neurons. Two mechanisms for the increased firing rate appeared to be a reduction in the slow component of the afterhyperpolarization (AHP) and a modest depolarization. Our observations suggest a signaling pathway via which signals acting on the lateral hypothalamus may influence the activity of the LC and thereby a variety of CNS functions related to noradrenergic innervation, including vigilance, attention, learning, and memory. Thus, the hypocretin innervation of the LC may serve to focus cognitive processes to complement hypocretin-mediated activation of autonomic centers already described.

The relevance of this work to sleep has been established by recent studies which indicate that ICV injections of the hypocretins promote wakefulness and decrease deep slow wave sleep and REM sleep (Hagan et al., 1999; Piper et al., 2000). Furthermore, two groups have recently shown that, in humans, the sleep disorder narcolepsy is associated with degeneration of the hypocretin/orexin neurons (Peyron et al., 2000; Thannickal et al., 2000). We have recently integrated this information into a general model for the role of the hypocretins in arousal state regulation (Kilduff and Peyron, 2000).

Gene Expression and Mammalian Hibernation. Very little information is available on molecular changes that correlate with hibernation state, and what has been done focused mainly on seasonal changes in peripheral tissues. The purpose of this study (O'Hara et al., 1999) was to characterize changes in gene expression in the brain of a seasonal hibernator, the golden-mantled ground squirrel, *Spermophilus lateralis*, during the hibernation season. We produced over 4000 reverse transcription-PCR products from euthermic and hibernating brain and compared them using differential display. Twenty-nine of the most promising were examined by Northern analysis. Although some small differences were observed across hibernation states, none of the 29 had significant changes. However, a more direct approach, investigating expression of putative hibernation-responsive genes by Northern analysis, revealed an increase in expression of transcription factors c-fos, junB, and c-Jun, but not junD, commencing during late torpor and peaking during the arousal phase of individual hibernation bouts. In contrast, prostaglandin D2 synthase declined during late torpor and arousal but returned to a high level on return to euthermia. Other genes that have putative roles in mammalian sleep or specific brain functions, including somatostatin, enkephalin, growth-associated protein 43, glutamate acid decarboxylases 65/67, histidine decarboxylase, and a sleep-related transcript SD464 did not change significantly during individual hibernation bouts. We did not observe a decline in total RNA or total mRNA during torpor as others had been previously hypothesized. Therefore, it appears that the dramatic changes in body temperature and other physiological variables that accompany hibernation involve only modest reprogramming of gene expression or steady-state mRNA levels.

We also examined expression of heat shock protein 70 (HSP70) in greater detail (Bitting et al., 1999). RNA transcripts of 2.7 and 2.9 kb hybridizing to an HSP70 cDNA were expressed in both brain and peripheral tissues of pre-hibernation euthermic animals; higher levels of expression were observed during the day than during nighttime samples. A decline in the expression of both transcripts occurred in all tissues examined during hibernation that remained low throughout the hibernation season, including the interbout euthermic periods and regardless of time of day. Quantitative comparisons showed pre-hibernation nighttime HSP70 expression to be as low as that observed during hibernation, despite the drastic increase in metabolic state and nearly 30 degrees C difference in body temperature. In contrast to HSP70, some mRNAs, such as beta-actin and HSP60, remained relatively constant, while others, such as glyceraldehyde 3-phosphate dehydrogenase, increased in specific tissues during the hibernation season. These results indicate that the expression of a highly conserved gene involved in protection from cellular stress, HSP70, can vary with an animal's arousal state.

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(8) Report of INVENTIONS:

L. de Lecea, T.S. Kilduff, P.E. Foye, P.E. Danielson, V.T. Gautvik, F.E. Bloom, K.M. Gautvik, and J.G. Sutcliffe. Hypothalamus-specific polypeptides. Provisional application submitted 8/2/96. International patent application filed 8/1/97. Publication of the international application with the international search report, 2/12/98.

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